



Circadian Rhythms of Urinary Cortisol Levels Vary Between Individuals in Wild Male Chimpanzees: A Reaction Norm Approach

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OPEN ACCESS

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Specialty section:

This article was submitted to
Behavioral and Evolutionary Ecology,
a section of the journal
Frontiers in Ecology and Evolution

Received: 12 March 2018

Accepted: 31 May 2018

Published: 19 June 2018

Citation:

Sonnweber R, Araya-Ajoy YG, Behringer V, Deschner T, Tkaczynski P, Fedurek P, Preis A, Samuni L, Zommers Z, Gomes C, Zuberbühler K, Wittig RM and Crockford C (2018) Circadian Rhythms of Urinary Cortisol Levels Vary Between Individuals in Wild Male Chimpanzees: A Reaction Norm Approach. *Front. Ecol. Evol.* 6:85. doi: 10.3389/fevo.2018.00085

Investigating the repeatability of trait variation between individuals, that is the amount of individual variation in relation to overall phenotypic variation, indicates an upper level of heritability and reveals whether a given trait may be subject to selection. Labile traits are characterized by high levels of flexibility and consequently low trait repeatability is expected. Indeed, research examining glucocorticoid levels in various non-mammal species found low repeatability scores. However, mammals may be different in this respect as (i) differential maternal care early in life has the potential to prime hypothalamic-pituitary-adrenal axis functioning and (ii) allelic variation affecting hypothalamic-pituitary-adrenal axis functioning has been reported. Individuals often differ from each other in average and/or plastic labile trait expression, two aspects that can be described using a reaction norm approach. Both consistent and flexible reaction norm expression has been argued to serve adaptive purposes, depending on the stability and predictability of environmental conditions. Here, we investigated both trait and reaction norm repeatability of urinary cortisol levels in wild adult male chimpanzees. To capture the expression of the circadian urinary cortisol rhythm of individual males over time, urine samples were collected throughout the day. In total data of 30 males collected over a period of 8 years were included in the dataset. No male was sampled over the whole 8-year period however. We found minor levels of trait repeatability but considerable reaction norm repeatability. This implies a minor role of genetic or priming factors on cortisol excretion, but reveals that males differ consistently in average urinary cortisol levels and the shape of the circadian urinary cortisol rhythm. Relating these results to fitness parameters will provide answers to questions on the adaptive value of reaction norm repeatability of this labile hormonal trait in the future.

Keywords: repeatability, reaction norm, repeatability of plasticity, circadian rhythm, glucocorticoids, individual differences

INTRODUCTION

Whether a trait varies more between or within individuals in a population has different ecological and evolutionary implications. Individual variation in a trait represents the raw material for natural selection and the stability of differences between individuals impacts the efficiency of adaptive responses to selection (Roche et al., 2016). Between individual variation in a trait is generated by differential exposure to relatively stable environmental factors, such as mothering styles (see for instance Liu et al., 1997; Francis and Meaney, 1999; Caldji et al., 2000), or by heritable differences between individuals (Dingemanse et al., 2010b; Forsman, 2015; Westneat et al., 2015). Individual *trait repeatability*, defined as the proportion of phenotypic variance explained by consistent between individual differences, indicates whether a trait can evolve through selection in principle (Dingemanse and Dochtermann, 2013; Baugh et al., 2014): traits with high repeatability—that is high relative between individual variation—can potentially be selected for if between individual variation is generated by genetic differences. Thus, measures of repeatability indicate an upper level of heritability. Generally, traits that are more plastic as their expression is more sensitive to external or internal circumstances, are predicted to have low trait repeatability (Bell et al., 2009). Therefore, when it comes to labile traits, differences between individuals may be more pronounced in the scope within which a trait is expressed, rather than in individual measurements. Average trait expression and/or, in the presence of environmental or internal gradients, plasticity of trait expression are commonly used in reaction norm approaches to estimate this scope of trait expression (e.g., Martin et al., 2011; Araya-Ajoy et al., 2015). Reaction norms are commonly characterized by an individual's average response to a mean centered environment (reaction norm intercept) and its level of plasticity (reaction norm slope) in response to that environmental gradient (Weis and Gorman, 1990; Via et al., 1995; Araya-Ajoy et al., 2015). Depending on the predictability of the environment and the time scale within which ecological conditions change, either highly flexible or less responsive phenotypes can be expected to cope better and represent a particular adaptive strategy (Dingemanse and Wolf, 2013; Botero et al., 2015). *Reaction norm repeatability*—that is how stable individuals are in their responses to environmental change over time—is expected only when individual attributes related to the costs and benefits of plasticity are stable over time. In the past years, research on average reaction norms and plasticity revealed that both can be subject to sexual and natural selection, and thus have important implications for environmental processes and trait evolution (Piersma and Drent, 2003; Nussey et al., 2005, 2007; Brommer, 2013; Dingemanse and Dochtermann, 2013; Westneat et al., 2015; Martin et al., 2017; Villegas-Ríos et al., 2017).

Currently, a strand of research in the field of evolutionary ecology focuses on examining levels of repeatability of labile traits, such as behavior or hormonal profiles (Nussey et al., 2007; Baugh et al., 2014; Westneat et al., 2015; Villegas-Ríos et al., 2017) as this is a promising avenue to tackle ultimate questions on selection pressures, adaptation, and fitness implications of

traits that are affected by ever changing environmental conditions (Brommer, 2013). A fair share of scientific effort in this line of research has focused on individual repeatability of glucocorticoid levels (e.g., Hruschka et al., 2005; Bonier et al., 2009; Thomas et al., 2009; Dingemanse et al., 2010a; Flinn et al., 2011; Ouyang et al., 2011a; Brommer, 2013; Cockrem, 2013; Narayan and Hero, 2013; Baugh et al., 2014; Hau et al., 2016). Glucocorticoids are a group of steroid hormones that function as physiological mediators to deal with metabolic demands arising from internal (e.g., metabolic) and external (e.g., ecological) circumstances (Sapolsky et al., 2000; Romero et al., 2009; Wingfield, 2013). So far, studies investigating repeatability in glucocorticoid levels in wild populations of various bird, fish, and reptile species have found low between individual variation in glucocorticoid levels, suggesting a negligible role of genetic or priming factors in glucocorticoid profiles (see for instance Ouyang et al., 2011a; Cockrem, 2013; Baugh et al., 2014; Lendvai et al., 2015; Small and Schoech, 2015; Hau et al., 2016). To our knowledge, in mammals between individual glucocorticoid repeatability has not been investigated so far. There is however evidence for some genetic predisposition of glucocorticoid excretion mechanisms (Linkowski et al., 1993; Oswald et al., 2004; Pflüger et al., 2016). Furthermore, a multitude of species has prolonged phases of intensive maternal care (e.g., Purvis and Harvey, 1995), rendering priming of glucocorticoid excretion patterns possible (e.g., Plotsky and Meaney, 1993, but see Aizer et al., 2015; Berghänel et al., 2016; Murray et al., 2016; McCormick et al., 2017). Therefore, exposure to differential levels of parental care and differences in genetic makeup could generate glucocorticoid trait repeatability among adult individuals in mammals.

While relatively little is known about glucocorticoid trait repeatability, repeatability of reaction norms is even less investigated (Araya-Ajoy et al., 2015; Hau et al., 2016). Studies investigating consistency in glucocorticoid responses between and within individuals are all derived from sampling plasma corticosterone in bird species and mostly share the caveat that the time frame over which measures are taken is relatively short, usually spanning days or months (e.g., Cockrem and Silverin, 2002; Romero and Reed, 2008). Results so far indicate that individuals are consistent in some aspects of their glucocorticoid excretion patterns but rather variable in other glucocorticoid response measures (e.g., high repeatability of stress-induced but not baseline glucocorticoid levels in Florida scrub jays, Rensel and Schoech, 2011). However, average glucocorticoid reaction norms and glucocorticoid plasticity as stable traits may be determined by the individual's genotype to a certain extent and thus be subject to selection (Rensel and Schoech, 2011).

Here, we investigated both trait and reaction norm repeatability of urinary cortisol (the main glucocorticoid excreted via urine in mammals) expression in males of a mammal species: namely two subspecies of chimpanzees (*Pan troglodytes verus* and *Pan troglodytes schweinfurthii*). Chimpanzees are an interesting species to study repeatability of urinary cortisol excretion and urinary cortisol reaction norms as infants are nursed by their mothers for around 4 years and carried and cared for even longer (Boesch and Boesch-Achermann, 2000), rendering maternal priming of the HPA axis

possible. Factors affecting glucocorticoid levels, such as circadian rhythms (Muller and Lipson, 2003), dominance rank (e.g., Muller and Wrangham, 2004; Anestis, 2005; Anestis et al., 2006; Muehlenbein and Watts, 2010), social dynamics (e.g., Fraser et al., 2008; Emery Thompson et al., 2010; Wittig et al., 2016), and maternal care (Murray et al., 2016) are well-studied in this species. This allows for an informed decision on potential control factors in statistical models and a sound biological interpretation of different patterns. We studied the circadian rhythm in urinary glucocorticoid excretion exhibited by chimpanzees (Muller and Lipson, 2003) and examined (i) trait repeatability of cortisol excretion, as well as (ii) the repeatability in circadian reaction norm parameters in adult male chimpanzees. The circadian cortisol excretion pattern (Kumar, 2017) is characterized by low levels of cortisol during the first half of the night, a continuous rise in cortisol levels toward the morning, and a pronounced increase at awakening, called the cortisol awakening response. Afterwards, throughout the day, cortisol levels decline, flattening in the afternoon, and reaching lowest levels in the evening. The circadian rhythm qualifies for the investigation of between individual variability in cortisol excretion for several reasons: (i) it represents a predictable internal gradient; (ii) due to ever changing social and environmental conditions within individual variation in cortisol levels along the gradient can be expected; (iii) lastly, the circadian rhythm in cortisol levels monitored over many years allows for detailed examination of repeatability estimates over shorter and longer periods. We predicted to find trait repeatability of cortisol phenotypes, which could either be due to differences in genetic makeup or because males are likely exposed differentially to factors that have the potential to prime HPA axis functioning, such as nutritional status of the mother during pregnancy (Edwards et al., 2002; Knackstedt et al., 2005) or postnatal maternal care (Murray et al., 2016). Furthermore, given the consistent results on reaction norm repeatability of corticosterone in birds (Hau et al., 2016), we expected to find that male chimpanzees are stable and consistently different from each other in their urinary cortisol reaction norms.

MATERIALS AND METHODS

Study Species and Study Sites

Data were collected at two sites on two subspecies of chimpanzees: *Pan troglodytes schweinfurthii* at the Budongo Conservation Field Station (1°43' N, 31°32' E) in Budongo forest, Uganda and *Pan troglodytes verus* at the Tai Chimpanzee Project (5°52'N, 7°20'E) in the Tai National Park, Ivory Coast. In Budongo forest the Sonso community was studied, whereas for Tai data on three groups (East, North, and South group) were available. Both are long-term field sites, with systematic observations starting in Tai in 1982 (Boesch and Boesch-Achermann, 2000), and in Budongo in 1990 (Reynolds, 2005). The two long-term field sites keep track of demographic changes in the chimpanzee communities and record rainfall and temperature measurements. Field assistants are employed at both sites to collect behavioral data and urine samples (for hormonal analyses). Additionally, data from a variety of

individual cross-sectional research projects were made available from the long-term databases of the respective field site.

For the current study, we used data on adult male chimpanzees of well-habituated groups spanning over a total of 8 years. We included data from periods for which both urine samples for non-invasive assessment of cortisol levels and behavioral data (pant grunt vocalizations to establish rank hierarchies, further details below) were collected. Data from the Tai field site used in this study stem from three different communities (East, North, and South group) and were collected between July 2004 and April 2010, and between October 2013 and May 2015. Data were sometimes collected during the same time periods across groups, but no community is represented over the whole study period. Data on the Budongo males were collected in one community (Sonso group) from June 2006 until November 2009. Male chimpanzees reach adulthood at 14 years. Ages in this study ranged from 15 to 49 years (Budongo: average age = 23.25, $SD = 9.26$, range = 15–49; Tai: average age = 23.55, $SD = 8.65$, range = 15–45). A detailed overview is given in Table S1 in the Supplementary Material. The chimpanzee communities differed regarding their group sizes and group composition (Table 1), ranging between an average of 9.47 ($SD = 1.37$) adult individuals in the North group of Tai to an average of 44.01 ($SD = 1.61$) adult individuals in the Sonso community. The ratio of the number of adult males to the number of adult females was relatively similar for all groups (ranging from 0.50–0.56), apart from the North group in Tai that had an average sex ratio of 0.36 ($SD = 0.12$).

Ethics Statement

At both sites, all methods of data collection were non-invasive. Permissions for the research were granted by the Ministries of Research and Environment of Ivory Coast and Office Ivorien des Parcs et Reserves for Tai, and Uganda Wildlife Authority and Uganda National Council for Science and Technology for Budongo. Furthermore, the Tai Chimpanzee Project and the Budongo Conservation Field Station approved all projects that made their data available for the current study. All aspects of the study comply with the ethics policy of both the Max Planck Society and the Department of Primatology of the Max Planck Institute for Evolutionary Anthropology, Germany (www.eva.mpg.de/primat/ethical-guidelines.html). Import and export certificates of urine sample shipments can be provided upon request.

Urine Sample Collection and Analysis

Examining urinary cortisol concentrations of free ranging animals is a widespread alternative to deriving cortisol concentrations from blood as this allows for capturing short-term changes in hormone concentrations using a non-invasive sampling regime (Hodges and Heistermann, 2003). While circulating hormones as measured in blood samples represent real-time fluctuations in endocrine activity, measures in urine usually show a time lag of 2–8 h (e.g., Miller et al., 1991; Bahr et al., 2000). Studies revealed that the circadian cortisol rhythm expressed in serum is comparable to the one observed in urine (Bartter et al., 1962; Kraan et al., 1998). Urine samples of the

chimpanzee populations were collected non-invasively and opportunistically when they could be assigned reliably to a specific male, as he was observed urinating (detailed descriptions of urine sample collection protocols in the Supplementary Material and in Deschner et al., 2003; Crockford et al., 2013). Several (18 out of 30) individuals died during the study period. However, only in three cases samples were collected within the month of death (in one case 2 out of 9 samples were collected in the month of death, in another case 2 out of 19 samples, and in the last case 3 out of 22 samples). The chimpanzee population in the Tai forest suffered outbreaks of Ebola and was exposed to Anthrax repeatedly in the past decades. Samples that were obtained during such outbreaks were not included in the current study. Samples were shipped frozen to the Laboratory of Endocrinology at the Max Planck Institute for Evolutionary Anthropology, Germany and stored at -80°C until analysis.

Liquid chromatography–tandem mass spectrometry was used to measure urinary cortisol levels (Hauser et al., 2008). Furthermore, each sample was analyzed for creatinine concentrations to correct cortisol measurements for water content in the urine, which varies with the time since the last urination and the level of an animal's hydration (Erb et al., 1970).

The Dataset

For our analyses, we had access to a total of 1,901 urine samples of 30 different adult males. Thirteen of those males were part of the Sonso community, Budongo Forest, Uganda, and 17 males inhabited the Tai forest, Ivory Coast. For Sonso a total of 617 urine samples was available (between 5 and 121 samples per male, average = 47.46, $SD = 35.07$). For Tai 1284 samples of adult males (between 10 and 218 samples per male, average = 75.53, $SD = 68.00$) were analyzed.

Whenever a urine sample was collected the time was noted. The first samples of the day were gathered at 05:30 in the morning and the last samples of the day were collected at $\sim 19:00$. In the current study, we studied the circadian rhythm in urinary cortisol excretion. Thus, the collection time data generated the gradient over which we investigated variation in cortisol. Most urine samples ($n = 1,628$) were collected between 8:00 and 17:00 with an average of 180.89 ($SD = 203.77$) samples per hour (Figure S1 shows a histogram of the number of samples collected binned into 1 h time intervals over the day in the Supplementary Material). A detailed overview of number of samples by time

interval and community or individual is given in Tables S2 and S3 in the Supplementary Material.

Extensive research has shown that a variety of social and ecological parameters correlate with excreted glucocorticoid levels (Beehner and Bergman, 2017). In this study, we controlled for the effects of such correlates to determine between individual variation in urinary cortisol levels independent of current social or environmental factors. To this end, we calculated several control variables: (i) in male chimpanzees, high social rank is associated with high glucocorticoid levels (Muller and Wrangham, 2004). To assess dynamic dominance relations amongst the males within each group, we used unidirectional submissive pant grunt vocalizations (Wittig and Boesch, 2003) that were collected during focal animal follows and with all occurrence sampling (Altmann, 1974). Data on submissive pant grunt vocalizations were then used to calculate daily Elo-rating scores (Neumann et al., 2011). Thus, for each urinary cortisol measurement of a given day a corresponding dominance score was assigned. All scores were standardized to values between 0 (lowest rank) and 1 (highest rank), so that scores were comparable between groups. (ii) HPA axis functioning changes in the course of the life-history of several primate species (e.g., Goncharova and Lapin, 2002; Seraphin et al., 2006). Therefore, we calculated the male's age (in years) at the time a given sample was collected. (iii) From the demographic data, we calculated the group size (including adult individuals only) and the ratio of the number of adult males to the number of adult females (Table 1), as larger groups as well as a skew toward more males and fewer females may increase competition and male-male aggression and therefore result in higher glucocorticoid levels (Pride, 2005; Raouf et al., 2006). (iv) Apart from these social influences, ecological factors are related to changes in glucocorticoid levels. Changes in rainfall and temperature are associated with differential energetic demands for an individual, resulting in changes in excreted glucocorticoid levels (Muller and Wrangham, 2004; Bales et al., 2006; Gesquiere et al., 2008; Emery Thompson et al., 2010; MacLarnon et al., 2015). Using the field sites' long-term temperature and rainfall data, we calculated average temperature and average precipitation per month for each year. Temperature varied relatively little within years (in Budongo: range = $21.15\text{--}24.07^{\circ}\text{C}$, average = 22.29°C , $SD = 0.77$); in Tai: range = $21.95\text{--}24.06^{\circ}\text{C}$, average = 23.19°C , $SD = 0.68$), precipitation in Budongo (average rainfall 5.09 mm,

TABLE 1 | Overview of data collection periods and demographic records by chimpanzee communities.

Site	Group identity	Data collection timespan	Number of data collection months	Number of adult males (mean \pm SD)	Number of adult females (mean \pm SD)	Mean (\pm SD) group size	Mean (\pm SD) sex ratio
Budongo	Sonso	2006–2009	39	15.77 \pm 1.48	28.24 \pm 1.55	44.01 \pm 1.61	0.56 \pm 0.07
Tai	East	2007–2015	34	5.67 \pm 0.63	11.05 \pm 2.39	16.72 \pm 2.65	0.53 \pm 0.11
Tai	North	2005–2008	13	2.41 \pm 0.51	7.06 \pm 1.48	9.47 \pm 1.37	0.36 \pm 0.12
Tai	South	2004–2015	44	7.86 \pm 1.06	16.33 \pm 2.53	24.18 \pm 1.82	0.50 \pm 0.15

The first column indicates whether a community (in the second column) belongs to the Tai or the Budongo population. In the third and fourth column, the years and total number of months for which data are available are indicated. The following columns show (i) the number of adult males, (ii) the number of adult females, (iii) the group size (including adult individuals only), and (iv) the sex ratio averaged over the data collection periods. The standard deviation (SD) is shown in brackets.

$SD = 2.25$), and Tai (4.99 mm, $SD = 3.81$) (more details in Table S4).

Data Preparations, Models, and Statistical Analysis

Urinary cortisol measures corrected for creatinine levels [LCMS measurement of urinary cortisol (pg/ μ l) divided by creatinine measurement (mg/ml) of the same sample] of adult male chimpanzees were log-transformed. The time a sample was collected was transformed into minutes since midnight and then mean centered and standardized two standard deviation units. Magnitude and sign of estimates of variance in reaction norm components are influenced by the measure and scale of the external or internal gradient used (Gelman, 2008). Therefore, we mean centered all control variables and standardized them to two standard deviation units (Araya-Ajoy et al., 2015).

We used random regression mixed-effect models (model structures also presented in Table S5 in the Supplementary Material) to study individual differences in cortisol levels throughout the day (Nussey et al., 2007). The response variable in all models was the log-transformed urinary cortisol measures corrected for creatinine. The following variables were added to all models: (i) since we could not expect a linear relationship between the time of day a sample was collected and urinary cortisol levels (Kumar, 2017), we added linear and quadratic effects of time of day to model the circadian rhythm in cortisol secretion; (ii) the dominance rank of each male on the day the urine sample was collected, (iii) the age of the male (in years) on the day the sample was collected (iv) the sex ratio of the day in the group, (v) group identity, (vi) monthly average rainfall in interaction with the time of day (higher glucocorticoid levels at times of high fruit availability (associated to higher precipitation) only in the morning, but not in the afternoon, (Muller and Lipson, 2003; Muller and Wrangham, 2004), and (vii) monthly average temperature measures. Community size was not included in the model as a control variable, as it correlated with the sex ratio (Spearman correlation: $r_s -0.06$, $p = 0.012$). To control for across years or months variation in social or ecological parameters not captured by the main effect control variables, we added random intercepts for years and months. As urinary cortisol measures derived from different research projects, we added a random intercept term for dataset to control for potential biases introduced by dataset variation.

To estimate *trait repeatability*, we added random intercepts for individuals to the first model (intercept model). A log-likelihood test comparing this model with a model excluding the random intercept for individual males revealed a significant effect of the individual male intercept (Chi-square = 11.57, $df = 1$, $p = 0.001$), indicating that males differ from each other in their average urinary cortisol expression. Since individuals were sampled repeatedly across the circadian gradient over years, we created a dummy variable (“male-year”), composed of male identity and sampling year, and included a random intercept for this variable too (the dummy variable “male year” encompassed 96 different male-year combinations). This allowed us to estimate short- (within year) and long-term (between years)

between individual variation in urinary cortisol levels (Araya-Ajoy et al., 2015). To calculate *reaction norm repeatability*, we build a second model (slope model): in addition to random intercepts for individuals and the dummy variable “male-year,” we included random slopes for the time and its quadratic term. A log-likelihood test comparing this model and a model excluding the random slopes for individual males and the dummy variable “male-year” revealed that males differed in the plasticity of their circadian cortisol levels (Chi-square = 20.38, $df = 10$, $p = 0.026$). Variance estimates of the random intercept and random slope models were used to calculate the repeatability of the reaction norm components.

Using the estimated variances of the random effects of these models, we calculated short- and long-term trait repeatability and repeatability of average reaction norms and reaction norm plasticity in urinary cortisol levels. **Table 2** gives an overview on how repeatability was calculated.

All data transformations, models, and repeatability analyses were done using RStudio (Version 1.0.136–© 2009–2016 RStudio, Inc.). Models were built and run using the lme4 package (Bates et al., 2014). Trait repeatabilities were estimate following Nakagawa and Schielzeth (2010) and reaction norm repeatabilities following Araya-Ajoy et al. (2015).

RESULTS

In a first step, we fitted a model including all main effects but excluding all random effects to test for potential model inflation. The model inflation factor was 1.47, indicating no issues with multicollinearity. Visual inspection of residuals’ histograms, qq-plots of residuals, and plots showing residuals against fitted values for the complete intercept and slope model suggested that all assumptions for Gaussian random regression mixed-effect

TABLE 2 | Calculating reaction norm (average reaction norm and plasticity) and trait repeatability in urinary cortisol levels (following Dingemanse and Dochtermann, 2013 and Araya-Ajoy et al., 2015).

Repeatability for	Calculated as
Trait repeatability of urinary cortisol levels across years	Intercept variance/total variance
Trait repeatability of urinary cortisol levels within years	(intercept variance + intercept male year variance)/total variance
Reaction norm repeatability of average reaction norms between males	Intercept variance/(intercept male + intercept male year)
Reaction norm repeatability of plasticity between males	Slope variance/(slope male + slope male year)

The first column indicates which repeatability was estimated and the second column shows how it was calculated. Trait repeatability in average urinary cortisol levels was calculated between and within years. Long-term repeatability indicates whether genetic or stable non-heritable factors influence excreted urinary cortisol levels, whereas the difference between short- and long-term repeatability indicates whether environmental factors are present that differentially affect individuals, but also vary between years. Repeatability of average reaction norms and reaction norm plasticity indicates the consistency of urinary cortisol reaction norms over time.

models were met. The results for the two models are shown in **Table 3** (further results for the control variables are presented in the Supplementary Material, Table S6). Exclusion of the quadratic effect of time of day did not alter the results (the model excluding the quadratic effect of time of day terms is presented in the Supplementary Material, Table S7).

Trait repeatability of urinary cortisol levels was 0.11 (lower confidence interval (LCI) = 0.07, upper confidence interval (UCI) = 0.17) between years and 0.19 (LCI = 0.14, UCI = 0.24) within years. This suggests low trait repeatability of urinary cortisol levels between and within years. The long-term repeatability score represents the fraction of variation caused by genes and stable environmental factors (permanent environmental effects). The difference between short- and long-term repeatability (0.07) points at some differences in average cortisol levels between individuals, generated by environmental conditions that differed between individuals and varied between years. A log-likelihood test comparing the random intercept and the random slope model revealed a significant impact of the random slopes (Chi-square = 20.38, $df = 10$, $p = 0.026$), suggesting that individuals' daily urinary cortisol excretion profiles differ from each other. Repeatability of reaction norm intercepts in urinary cortisol levels was 0.81 (LCI = 0.71, UCI = 0.88) and repeatability of urinary cortisol plasticity was 0.31 (LCI = 0.20, UCI = 0.43) for the linear term and 0.65 (LCI = 0.65, UCI = 0.65) for the quadratic term. Thus, males differed relatively consistently in average reaction norms and reaction norm plasticity in urinary cortisol levels across years. A visual presentation of individual male reaction norms is presented in **Figure 1** (for the circadian gradient).

DISCUSSION

The populations of wild male chimpanzees in our study exhibited relatively low trait repeatability in urinary cortisol levels. Across and within year repeatability was comparable to repeatability scores reported in other studies on between individual variation in glucocorticoid levels (e.g., Baugh et al., 2014) and behavioral traits (reviewed by Bell et al., 2009). While the studies revealing similar levels of between individual glucocorticoid repeatability as our study used comparably short sampling periods (spanning over days or months), the data presented here accumulated over several years. Short-term repeated sampling can lead to higher repeatability estimates compared to longer sampling periods (Hau et al., 2016). Thus, trait repeatability in adult male chimpanzee urinary cortisol levels can be regarded as being higher than expected.

When comparing short- and long-term trait repeatability of cortisol levels in adult male chimpanzees, we found that short-term repeatability was in fact higher within years (0.19) than between years (0.12). On the one hand this indicates the necessity to consider the duration of sampling regimes when interpreting results, in particular when data are available for a short period of time only. On the other hand, it points to another interesting aspect: there seemed to be environmental factors that affected

TABLE 3 | Results for the random effects of the fitted models.

	Random intercept model	Random slope model
Fixed effects	β (95% CI)	β (95% CI)
Intercept	4.23 (3.70, 4.75)	4.23 (3.73, 4.74)
Time	-0.59 (-0.65, -0.53)	-0.56 (-0.67, -0.47)
Time squared	-0.22 (-0.34, -0.09)	-0.22 (-0.39, -0.06)
Dominance rank	0.09 (-0.05, 0.25)	0.09 (-0.06, 0.24)
Sex ratio	-0.29 (-0.45, -0.14)	-0.29 (-0.44, -0.13)
Age at sample	0.24 (0.06, 0.43)	0.17 (-0.02, 0.35)
Group identity	-0.39 (-1.09, 0.24)	-0.38 (-1.02, 0.27)
Rainfall	-0.03 (-0.12, 0.05)	-0.03 (-0.11, 0.06)
Temperature	-0.14 (-0.25, -0.02)	-0.14 (-0.25, -0.02)
Rainfall * time	0.11 (0.00, 0.21)	0.08 (-0.02, 0.20)
RANDOM EFFECTS		
Variance male-ID intercept	0.06 (0.04, 0.10)	0.18 (0.10, 0.29)
Variance male-ID slope for time		0.02 (0.01, 0.03)
Variance male-ID slope for time squared		0.02 (0.02, 0.02)
Variance male-year intercept	0.04 (0.03, 0.05)	0.04 (0.03, 0.05)
Variance male-year slope for time		0.05 (0.04, 0.07)
Variance male-year slope for time squared		0.01 (0.01, 0.01)
Variance year	0.01 (0.00, 0.02)	0.01 (0.00, 0.01)
Variance month	0.04 (0.02, 0.08)	0.05 (0.02, 0.10)
Residual variance	0.38 (0.36, 0.41)	0.37 (0.35, 0.40)

Variances of the "random intercept model" were used to estimate trait repeatability of urinary cortisol levels. Variances of the "random slopes model" were used to estimate repeatability of urinary cortisol reaction norms (average reaction norm and plasticity). Results are based on 1,901 urine samples of 30 adult males. These data stem from ten different years and from seven different datasets. The dummy variable "male year" encompassed 96 different male-year combinations.

individual adult males differently and that varied more strongly between years than within years. A challenge for future research will be to determine which factors these are and what their biological or ecological implications may be. Similar to trait repeatability scores, short-time intervals of repeated sampling tend to reveal higher repeatability for reaction norms of labile traits than examinations over longer periods (Hau et al., 2016). Therefore, making use of long term data collection and extensive repeated sampling has the potential to gain more insight into the level of reaction norm consistency in glucocorticoid levels and plasticity (but see Martin et al., 2011; van de Pol, 2012). For a range of mammal species, particularly non-human primates, such data are becoming available thanks to the continuous scientific work at various long term field sites, such as the Tai Chimpanzee Project (<https://www.eva.mpg.de/primat/research-groups/chimpanzees/field-sites/tai-chimpanzee-project.html>).

Although it has been recognized that there is considerable variation in glucocorticoid excretion patterns, research on reaction norm variation is still rather limited (Hau et al., 2016). In the present study, we found considerable repeatability of

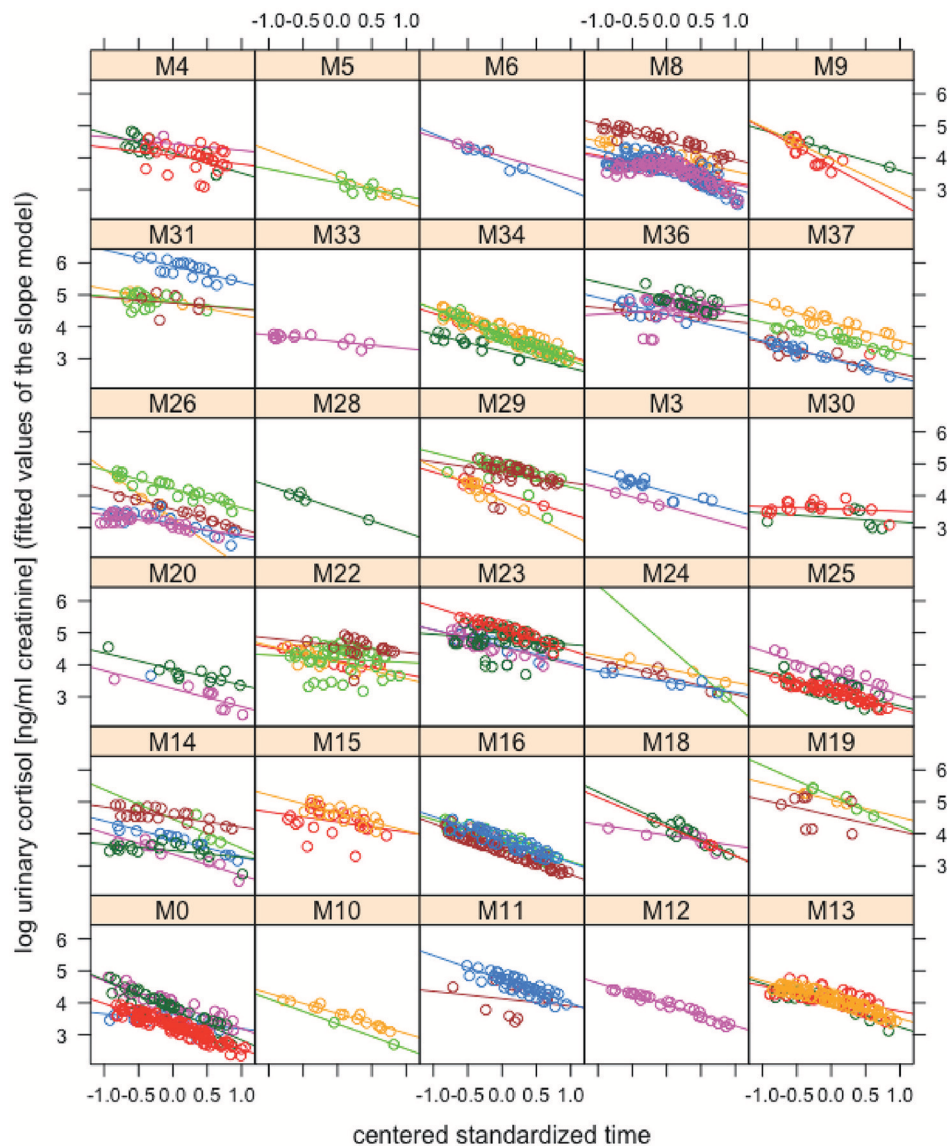


FIGURE 1 | Reaction norms of urinary cortisol levels. Log-transformed urinary cortisol concentrations (ng/ml creatinine) are plotted against the mean centered standardized time gradient based on the fitted values obtained from the “slope model.” Each box contains data of one individual. Differently colored lines and dots in individual boxes represent data from different “male-years.” While all males show a steady decrease in urinary cortisol levels across the day, they differ from each other in urinary cortisol intercepts and slopes: e.g., male M31 (first box in the second row from top) has higher average urinary cortisol levels than male M34 (third box in the second row from top) and male M22 (second box in the fourth row from top) shows a relatively flat decrease in urinary cortisol levels as compared to male M0 (first box in the bottom row). Crucially, these differences in average expression and shape of the circadian urinary cortisol rhythm between males show consistency across years (repeatable across years).

both reaction norm intercept (0.81) and reaction norm plasticity (0.31 and 0.65 for slopes over the linear and the quadratic term for time) in urinary cortisol levels. This result shows that adult male chimpanzees are relatively consistent in their cortisol excretion patterns—at least over the circadian gradient—and that individuals differ consistently from each other in both average cortisol reaction norms and in the shape of their circadian urinary cortisol rhythm. As can be seen in **Figure 1**, all males show a decrease in urinary cortisol levels across the day. This is consistent with what would be expected from the literature

(Muller and Lipson, 2003; Kumar, 2017). Males differ from each other in the average expression and steepness of urinary cortisol level decreases across the day however. Crucially, those differences between males show consistency across years: males expressing a steep decrease of urinary cortisol levels throughout the day in 1 year, will express a similar pattern in another year (for instance male M0 in **Figure 1**). Likewise, a male showing a comparably flat decrease in urinary cortisol levels throughout the day in 1 year, will also do so in another year (for instance male M30 in **Figure 1**).

Estimating variation in reaction norm slope parameters requires repeated measures of the phenotype across an environmental gradient, but the exact level of replication depends on the particularities of each study. To check for the robustness of our approach we used statistical simulations tailored to estimate the probability to detect significant results in a scenario where our data would show no variation in reaction norm slopes. We simulated data with the same structure as the actual data, but in the simulated dataset we assumed absence of variance in urinary cortisol levels in the slopes for individual males and the dummy variable “male-year.” If the results we reported were not spurious, the variance estimated from the model applied to the simulated data should be smaller than the variance estimated from the actual data (see Araya-Ajoy and Dingemanse, 2017 for a similar approach). We ran 1,000 simulations and estimated the probability that the observed variance estimated for the actual data was equal or smaller than the variance estimated for the simulated data (with no variation in reaction norm slopes). Furthermore, to estimate potential bias due to our unbalanced dataset (some males are represented with more samples, over more years, than others, also apparent in **Figure 1**), we simulated data with the exact structure as the actual data and used the parameters of the model results. Thus, analysis of the simulated data should provide the same estimates as the models applied to the real data. The deviations from the parameters applied to the real data and the simulated data are the bias associated to the sample size and structure of the data. Levels of bias due to data structure and sample size were low (deviations of parameter estimates of simulated and actual data: time slope in individual = 0.22; quadratic time term in individual = 0.03; time in male year = 0.19; quadratic time term in male year = 0.04). We found that the probability that our estimates were the result of the data structure and chance was always lower than 0.05. Therefore, the parameter estimates of variance in slopes are significant but their values need to be taken with care.

What may be the biological implications of such between individual differences? Differences in the circadian rhythm of cortisol have been reported for different chronotypes for instance: in humans, evening-types show lower morning arousal and a delay in peak cortisol levels in the morning as compared to morning types (e.g., Bailey and Heitkemper, 1991). Also, differential exposure to stressors has been associated to differences in circadian patterns of cortisol in humans (e.g., Adam and Gunnar, 2001). In male chimpanzees, different life-history strategies may be associated with the patterns observed: males may face different levels of (inter- and intra-group) competition and aggression, and consequently be exposed to different (levels) of stressors, which may be reflected in different urinary cortisol reaction norms. A next step would be to compare repeatability of urinary cortisol levels over other gradients and contexts, such as gradients of differential food availability or social group stability. Two main scenarios of how individuals respond to diverse gradients are possible: (i) individuals could show very consistent glucocorticoid responses to any given stimulus that affects its metabolic rate, and therefore, they could express similar levels of average and plastic glucocorticoid responses across conditions. (ii) Variation in glucocorticoid

levels could also result in divergent glucocorticoid responses to different conditions if differences in neural processing of (experienced or anticipated) external and internal stimuli emerge (e.g., Hau et al., 2016).

While the mechanisms behind glucocorticoid excretion patterns are relatively well-studied, comparably little is known about the ultimate implications of different glucocorticoid profiles (Beehner and Bergman, 2017). Evaluating consistency in individual cortisol levels promises to reveal links between a hormonal trait and fitness measures. For instance, Ouyang et al. (2011b) found that house sparrows that had high corticosterone levels before egg laying and low corticosterone levels during breeding exhibited enhanced reproductive success. Thus, plasticity in corticosterone levels was related to increased fitness. So far, a couple of studies have revealed relationships between hormones, behavior, and fitness in wild animals and suggest a genetic basis of corticosterone profiles (for a review see Zera et al., 2007). Long-term studies using a reaction norm approach in wild populations have great potential to shed light on fitness implications of between and within individual variation in glucocorticoid profiles. Dingemanse et al. (2010b) proposed to assess the covariance of fitness proxies with intercepts and slopes of reaction norms. This will further our understanding of the relationship between fitness outcomes and glucocorticoid excretion patterns. Glucocorticoids are amongst the best studied hormones in behavioral endocrinology, probably due to the multitude of processes that glucocorticoids are involved in and owing to well-established methods for analysis (Schwarzenberger et al., 1996; Heistermann, 2010; Creel et al., 2013; Murtagh et al., 2013; Behringer and Deschner, 2017). However, particularly in non-human primates, studies investigating the relationship between fitness and glucocorticoids are lacking (Beehner and Bergman, 2017). Combining approaches from evolutionary ecology and behavioral endocrinology may prove a fruitful path in this endeavor. Here, we have aimed to take a first step into this direction by showing that male chimpanzees are consistent in the expression of their urinary cortisol reaction norms. The crucial next step will be to relate this variation in a physiological trait to fitness parameters such as reproductive success, survival, or morbidity. We hope that research uniting patterns of variation in physiological traits and fitness measures will accelerate further.

Disentangling genetic from environmental factors generating between male variation in urinary cortisol levels and reaction norms will be crucial for making inferences about the role of selection in the expression of different urinary cortisol excretion patterns: only genetically-determined variation represents a basis for selection forces to act on and therefore repeatability estimates cannot be used as forthright proxies for heritability. However, Dochtermann et al. (2015) pointed out that whenever repeatability and heritability are assessed for the same dataset, the relative influence of additive genetic variation can be estimated. Using this rationale, the authors showed that, in the case of animal personalities, heritable differences likely play an important role and that repeatability estimates may allow for conclusions about the role of selection processes in the emergence of between individual variation in personality. Similarly, with a growing number of studies investigating the

heritability and repeatability of physiological measures, such as glucocorticoids, questions about the role of selection will likely be answered in the future.

AUTHOR CONTRIBUTIONS

RS and VB: conception of the study; TD, AP, LS, ZZ, CG, RW, and CC: data collection; RS and YA-A: statistical analysis and interpretation; RS and VB: drafting of the manuscript; RS, YA-A, VB, TD, PT, PF, AP, LS, ZZ, CG, KZ, RW, and CC: revising manuscript and final approval.

FUNDING

This research was funded by The European Research Council (ERC) under the European Union's Horizon 2020 research and innovation program (Grant Agreement No 679787), awarded to CC and the Research Council of Norway (SFF-III 223257/F50).

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ACKNOWLEDGMENTS

We like to thank Alexander Mielke for making the R code to calculate ELO ranks available. We thank the Ministries for Research and Environment of Ivory Coast, the Office Ivorien des Parcs et Reserves, the Uganda Wildlife Authority, and the Uganda National Council for Science and Technology for permission to conduct research. We are grateful to the Centre Suisse de Recherches Scientifiques, the staff members of the Tai Chimpanzee Project and the Budongo Conservation Field Station for continuous support and collection of long-term data and the members of the DFG Researcher Unit (FOR 2136) Sociality and Health in Primates for discussions.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fevo.2018.00085/full#supplementary-material>

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